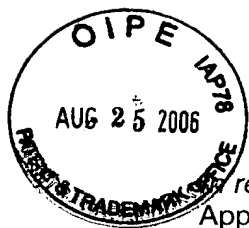


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Patent**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

re of Application of: Alan R. Davis *et al.*
Application No.: 09/457,421 Group Art No.: 1648
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For: RECOMBINANT ADENOVIRUS VACCINES
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SECOND AMENDED APPEAL BRIEF

This Amended Appeal Brief is submitted in response to the July 31, 2006 Notification of Non-Compliant Appeal Brief (37 CFR 41.37(d)), which stated that the Amended Appeal Brief mailed July 14, 2006 was missing the eighth and ninth listed items. Appellants respectfully submit that this Second Amended Appeal Brief now contains these two items and, therefore, is in compliance with all the formal requirements of 37 CFR 41.37. Appellants further respectfully request that the Board of Patent Appeals and Interferences reverse the Examiner's rejection of the claimed subject matter.

CERTIFICATE OF MAILING 37 CFR §1.10

I hereby certify that this paper and the documents referred to as enclosed therein are being deposited with the United States Postal Service on the date written below in an envelope as "Express Mail Post Office to Addressee" Mailing Label Number ER672187258US addressed to the Mail Stop Appeal Brief-Patents, Commissioner for Patents, PO Box 1450, Alexandria, VA 22313-1450.

August 25, 2006
Date

Alan M. Gordon

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I. REAL PARTY IN INTEREST

The real party in interest in the above-identified application is Wyeth, the assignee of all right, title and interest. The Assignment was recorded by the assignment division of the U.S. Patent and Trademark Office on February 11, 2004, on Reel 014335, Frame 0946.

II. RELATED APPEALS AND INTERFERENCES

No other appeals or interferences are known to Appellants, Appellants' legal representative, or the assignees, which will directly affect, be directly affected by, or have a bearing on the Board's decision in the pending appeal.

III. STATUS OF CLAIMS

Claims 1-16 were canceled in a Preliminary Amendment submitted September 04, 2003. Claims 17-25 were canceled in an Amendment and Response to Restriction Requirement submitted October 14, 2003. Claims 34-36 were amended and new claim 40 was added in an Amendment submitted February 18, 2004. Claims 26 and 30 were amended and new claim 41 was added in an Amendment submitted June 18, 2004. Claims 26, 28, 35, 36, and 39 were amended and claims 27 and 41 were canceled in the Amendment submitted December 20, 2004.

Claims 26 and 28-40 are pending in the application, each of which stand rejected under 35 U.S.C. § 103(a). The pending claims are all on appeal and are set forth in **Appendix A** of this Brief.

IV. STATUS OF THE AMENDMENTS

A Final Office Action was mailed November 02, 2005, rejecting all pending claims of the instant application. A Notice of Appeal was filed on January 30, 2006, and received by the U.S. Patent Office on January 30, 2006.

V. SUMMARY OF THE CLAIMED SUBJECT MATTER

The present invention is based, at least in part, on the discovery and development of novel methods for producing an immune response against HIV-1 infection in a human (see, *e.g.*, Independent Claim 26 in Appendix A).

Appellants' methods for producing an immune response against HIV-1 infection in a human (hereinafter, "methods") comprise (1) administering to the human an intranasal or intramuscular dosage of a recombinant adenovirus comprising an expression cassette containing a promoter, a nucleic acid sequence encoding the HIV-1 gp160 or gp120 polypeptide sequence and a polyadenylation sequence (see, *e.g.*, page 5, lines 21-26; page 9, line 22 through page 10, line 12; and page 11, line 16 through page 12, line 25) and (2) administering to the human one or more intranasal or intramuscular booster dosages of the recombinant adenovirus (see, *e.g.*, page 5, line 28 through page 6, line 1; and page 6, lines 8-9).

Appellants' methods also pertain to administering one or more intramuscular injections of an HIV-1 gag polypeptide, an HIV-1 env polypeptide, or a combination thereof (see, *e.g.*, page 6, lines 10-12; page 6, lines 18-19; and page 12, line 26 through page 13, line 3). The claimed subject matter further pertains to the recombinant adenovirus used in the methods for inducing an immune response, wherein the adenovirus is serotype 4 (Ad4), serotype 5 (Ad5) or serotype 7 (Ad7) (see, *e.g.*, page 6, lines 1-2; page 6 line 21 through page 7, line 21).

Appellants' methods also pertain to expression cassettes, which in addition to containing a promoter, a nucleic acid sequence encoding the HIV-1 gp160 or gp120 polypeptide and a polyadenylation sequence, further comprise the HIV-1 *rev* gene (see, *e.g.*, page 7, lines 9-12; and Example 1, page 25, line 28 through Example 4, page 10, line 28). The methods also pertain to the gp160 sequence derived from an HIV-1 MN or LAV strain (see, *e.g.*, page 6, lines 5-6) and replacing the gp160 (*i.e.*, env) sequence with a sequence encoding the gag-pro region of HIV-1 (see, *e.g.*, page 6, lines 6-7; and Example 1, page 8, line 28 through page 9, line 15).

Appellants' methods further pertain to specific intranasal and intramuscular (priming and boosting) dosages of the recombinant adenoviruses (see, *e.g.*, page 6, lines 12-18) and subunit polypeptide (boosting) dosages (see, *e.g.*, page 6, lines 18-19) for use in producing an immune response against HIV-1 in a human. Additionally, the methods of the invention pertain to adenoviruses comprising E1 gene deletions, E3 gene deletions or a combination of E1/E3 gene deletions (see, *e.g.*, page 5, lines 4-6).

VI. GROUND OF REJECTION TO BE REVIEWED ON APPEAL

Appellants present the following issues for review:

- A.** Whether claims 26 and 28-40 are unpatentable under 35 U.S.C. § 103(a) over Hung *et al.* (*Nat. Immun. Cell Growth Regul.*, 9:160-164, 1990) in view of Davis *et al.* (U.S. Patent No. 4,920,209).

- B.** Whether claims 26, 28-31 and 33-40 are unpatentable under 35 U.S.C. § 103(a) over Chanda *et al.* (*Int. Rev. Immunol.*, 7(1):66-77, 1990) in view of Davis *et al.* (U.S. Patent No. 4,920,209).

VII. ARGUMENTS

Claims Rejected Under 35 U.S.C. §103(a) over Hung et al. or Chanda et al. in view of Davis et al.

Claims 26 and 28-40 stand rejected under 35 U.S.C. § 103(a) over Hung *et al.* (*Nat. Immun. Cell Growth Regul.*, 9:160-164, 1990; copy included in Appendix B) in view of Davis *et al.* (U.S. Patent No. 4,920,209; copy included in Appendix B).

Claims 26, 28-31 and 33-40 stand rejected under 35 U.S.C. § 103(a) over Chanda *et al.* (*Int. Rev. Immunol.*, 7(1):66-77, 1990; copy included in Appendix B) in view of Davis *et al.* (U.S. Patent No. 4,920,209).

The Examiner acknowledges that neither Hung *et al.* nor Chanda *et al.* teach or suggest the presently claimed prime-boost methods of the invention. The Examiner then asserts:

"However, this is an obviousness rejection. Thus, neither Hung *et al.* nor Chanda *et al.* have to teach or describe the claimed method to render the claimed method unpatentable."

Nonetheless, the Examiner has ignored a crucial requirement for the assertion of an obviousness rejection, namely, that there be some teaching or suggestion that the references cited be combined. The leading decisions of the Court of Appeals for the Federal Circuit make it clear that the combination of the references relied upon by the Examiner is legally impermissible. The impropriety of such a combination was set forth by the Court of Appeals for the Federal Circuit in *In re Stencel*, 4 USPQ2d 1071, 1073 (Fed. Cir. 1985):

"Nor is obviousness established by combining the teachings of the prior art to produce the claimed invention, absent some teaching or suggestion that the combination be made."

The Federal Circuit cases reaffirming this important point are legion, going back more than twenty years. See also, for example, *Ashland Oil, Inc. v. Delta Resins & Refractories, Inc.*, 227 USPQ 657, 664 (Fed. Cir. 1985); *ACS Hospital Systems, Inc. v. Montefiore Hospital*, 221 USPQ 929, 933 (Fed. Cir. 1984); *In re Gordon*, 221 USPQ

1125, 1127 (Fed. Cir. 1984); *SmithKline Diagnostics Inc. v. Helena Laboratories Corp.*, 8 USPQ2d 1468, 1475 (Fed. Cir. 1988); *In re Bond*, 15 USPQ2d 1566, 1568 (Fed. Cir. 1990); *In re Oetiker*, 24 USPQ2d 1443, 1446 (Fed. Cir. 1992); *Carella v. Starlight Archery*, 231 USPQ 644, 647 (Fed. Cir. 1986). Such a teaching or suggestion is sorely lacking here.

The Examiner further alleges, however, that based on the knowledge generally available to one of ordinary skill in the art, it would have been *prima facie* obvious for one of skill to have used various methodologies of administering the composition, such as in a prime and boost methodology. The Examiner states that: "One of ordinary skill in the art at the time the invention was made would have had a reasonable expectation of success in doing so because in order to arrive at a administration methodology that would render the most beneficial immune response against HIV-1 infectivity, one of ordinary skill in the art would have necessarily used various methodology of administering the composition as part of routine experimentation" [emphasis added] (*e.g.*, see Final Action dated November 02, 2005; page 4, first paragraph, last sentence). Appellants respectfully disagree, and point out that the Examiner has cited no references to the prior art to support her conjecture.

Additionally, the Examiner rejects certain dependent claims directed to deletions in the adenovirus E1 and/or E3 genes (*e.g.*, claims 38-40, which depend from independent claim 26) over Hung *et al.* or Chanda *et al.* in view of Davis *et al.* The Examiner states that Davis *et al.* teaches that a deletion in the early region 1 (E1) or early region 3 (E3), or both, may be deleted to maximize the length of the foreign nucleic acid sequence that can be inserted into the vector. The Examiner therefore contends that one of skill in the art would have been motivated to combine the teachings of Davis *et al.* with Chanda *et al.* or Hung *et al.* to practice the presently claimed methods. Appellants respectfully disagree.

Independent claim 26 of the present invention is directed to a method for producing an immune response against HIV-1 infection in a human comprising (1)

administering an intranasal or an intramuscular dosage of a recombinant adenovirus comprising an expression cassette containing a promoter, a nucleic acid sequence encoding the HIV-1 gp160 or gp120 polypeptide sequence and a polyadenylation signal sequence and (2) administering one or more intranasal or intramuscular booster dosages of the recombinant adenovirus. Claims 28-40 are dependent from claim 26.

Appellants' data clearly demonstrate that the claimed methods, using the adenovirus-HIV constructs, are immunogenic in both dogs (Treatment Regimen 4, pages 24-25 and Treatment Regimen 5, pages 25-27) and non-human primates (*i.e.*, chimpanzees) (Treatment Regimens 1-3, pages 13-23 and Treatment Regimen 6, pages 27-32). Furthermore, Appellants' data was the first to demonstrate that the claimed methods (*i.e.*, a prime-boost regimen) protect non-human primates against HIV-1 challenge (Treatment Regimen 6). For example, page 38 of the specification summarizes the results of Treatment Regimen 6, stating that:

The intranasal administration of the Ad-env recombinants (particularly Ad7-env_{MN}, Ad5-env_{MN}, Ad4-env_{MN}, or a combination thereof) elicited the production of neutralizing antibodies against HIV-1. Neutralizing antibodies were produced following the first administration of the Ad-env recombinants, and the titer was increased through the use of one or more booster intranasal immunizations with the Ad-env recombinants. Antibody response to both MN and SF2 strains of HIV was further boosted through the administrations of one or more inoculations with an env (gp120) subunit antigen preparation (particularly gp120_{SF2}). Most importantly, protection against HIV-1 infection was demonstrated following the administration of the Ad-env/subunit booster treatment regimen. [emphasis added]

As set forth in detail below, it will become clear that neither Hung *et al.* reference or Chanda *et al.* reference teach, describe, or suggest the presently claimed adenovirus vectored prime-boost methods for producing an immune response against HIV-1 infection, as described above and hereinafter in detail.

The Hung et al. and Chanda et al. References

The Hung *et al.* reference describes a recombinant adenovirus type 7 (Ad7) construct comprising a gene expressing either the hepatitis B surface antigen (HBsAg) or the human immunodeficiency virus type 1 envelope glycoprotein (*env*). Hung *et al.* demonstrate that the Ad7-HBsAg construct is propagated in cultured cells and that propagation of the Ad7-*env* construct in cultured cells required co-infection with an Ad7 construct expressing the HIV-1 *rev* gene. Hung *et al.* further demonstrated that intra-tracheal (*i.e.* oral delivery) inoculation of dogs with the Ad7-HBsAg construct induced an antibody response against the hepatitis B antigen. Similar studies in dogs were not performed with the Ad7-*env* (*i.e.*, HIV-1) construct.

Thus, there is no data (either *in vitro* or *in vivo*) in the Hung *et al.* reference to suggest that the Ad7-*env* would in fact elicit an immune response in dogs or any other mammals. Furthermore, Hung *et al.* does not teach or suggest administering the recombinant adenovirus (*i.e.*, a Ad7-HbsAg or a Ad7-*env*) *via* an intranasal or intramuscular administration route, nor does Hung *et al.* teach or suggest administering one or more intranasal or intramuscular booster dosages of the recombinant adenovirus as presently claimed. Appellants therefore assert that Hung *et al.* does not teach nor describe the presently claimed prime-boost method for producing an immune response against HIV-1 infection in a human.

The Chanda *et al.* reference describes a recombinant Ad7 construct comprising the HIV-1 *env* gene (Ad7-*env*), the major late promoter (MLP), the tripartite leader (TPL) and a poly-A sequence. Chanda *et al.* also describe a second Ad7 construct comprising both the *env* and *rev* genes (Ad7-*rev-env*). Chanda *et al.* demonstrated that the *env* protein is expressed in A549 and HEK cells infected with Ad7-*env* or Ad7-*rev-env*. In contrast, Chanda *et al.* only immunized dogs orally with an Ad7 construct expressing hepatitis B surface antigen (HbsAg, *i.e.*, an Ad7-HbsAg construct) and observed an anti-HBs response, stating that “the data demonstrate that under semi-permissive conditions, adenovirus vectors may induce seroconversion to products of

foreign gene inserts” [emphasis added]. Chanda *et al.* did not perform a similar study in dogs with the Ad7-env or Ad7-env-rev constructs (*i.e.*, HIV-1 constructs).

Thus, there is no data (either *in vitro* or *in vivo*) in the Chanda *et al.* reference to suggest that the Ad7-env would in fact elicit an immune response in dogs or any other mammals. Furthermore, Chanda *et al.* does not teach or suggest administering one or more intranasal or intramuscular booster dosages of the recombinant adenovirus as presently claimed. Appellants further contend Chanda *et al.* particularly teaches away from the present invention, stating that “the dog is only semi-permissive for Ad7 replication and requires intratracheal [oral] administration of high doses of the virus to induce immune responses” (Chanda *et al.*, page 74, second paragraph, third sentence) [emphasis added]. Appellants therefore assert that Chanda *et al.* does not teach nor describe the presently claimed adenovirus vectored prime-boost method for producing an immune response against HIV-1 infection in a human.

However, throughout the prosecution of the present application (including the most recent Final Action), the Examiner has repeatedly advanced and maintained the same “obvious to experiment” rejection of the pending claims over Hung *et al.* and Chanda *et al.*

“Obvious-to-Try” Is Not an Appropriate Test of Obviousness

In the Official Action dated September 22, 2004 (*e.g.*, see page 6, first full paragraph through page 7, first paragraph and page 10, first full paragraph through page 12, first paragraph), the Examiner alleged that one of skill in the art, at the time the invention was made, would have been motivated by the teachings of the Hung *et al.* or Chanda *et al.* to “experiment with various methodology of administering the composition, such as a prime and boost methodology. One of ordinary skill in the art at the time the invention was made would be motivated to practice said methodology that would render the most beneficial immune response against HIV-1 infectivity. One of ordinary skill in the art at the time the invention was made would have had a

reasonable expectation of success for doing so because such experimentation is routine experimentation to arrive at an optimal administration methodology" [emphasis added].

In the same Action, the Examiner further alleged that one of skill in the art would have been motivated by the teachings of the Hung *et al.* or Chanda *et al.* to (a) "experiment" with and administer booster env and/or gag protein subunits, (b) "experiment" with the subunit antigen dosage amounts, (c) "experiment" with the location of the rev gene and insert the rev gene in frame and after the env gene but before the poly-A signal sequence and (d) select the appropriate HIV-1 strains.

Appellants assert that this invitation to "experiment" argument made by the Examiner is an "obvious-to-try" rejection, which is not the standard for obviousness under 35 U.S.C. § 103. "Obvious-to-try has long been held not to constitute obviousness". *In re O'Farrell*, 853 F.2d 894, 903, 7 U.S.P.Q.2d 1673, 1680-81 (Fed. Cir. 1988).

An obvious-to-try situation exists when a general disclosure may pique the scientist's curiosity, such that further investigation might be done as a result of the disclosure, but the disclosure itself does not contain a sufficient teaching of how to obtain the desired result, or that the claimed result would be obtained if certain directions were pursued. *In re Eli Lilly & Co.*, 902 F.2d 943, 14 U.S.P.Q.2d 1741, 1743 (Fed. Cir. 1990).

More specifically, as was stated in *In re Dow Chemical*, 5 USPQ2d 1529, 1532 (Fed.Cir. 1988), "obvious to experiment" is not an appropriate test of obviousness:

The PTO presents, in essence, an 'obvious to experiment' standard for obviousness. However, selective hindsight is no more applicable to the design of experiments than it is to the combination of prior art teachings. There must be a teaching or suggestion in the art for selecting the procedure used, other than the knowledge learned from the applicant's disclosure.

Appellants assert that given the state of the HIV arts art at the time of the present invention (*i.e.*, 1992), a person of skill in the art would not have been motivated by the Hung *et al.* reference or the Chanda *et al.* reference (which provide no data with regard to the Ad7-HIV-1 *env* immunogenicity) to administer this construct to a human to induce an immune response against HIV-1 infection. Furthermore, in the absence of Appellants' data set forth above, one of skill would not have had a reasonable expectation of success based on the disclosure of either the Hung *et al.* reference or the Chanda *et al.* reference.

Appellants therefore assert that neither Hung *et al.* nor Chanda *et al.*, taken alone or in combination with Davis *et al.*, render the presently claimed invention obvious. In fact, as set forth below, the Examiner had previously confirmed Appellants' position (*i.e.*, that the presently claimed invention is not obvious), wherein the Examiner stated that there are numerous scientific obstacles related to HIV infection and treatment which "establish that the contemporary knowledge in the art would not allow one skilled in the art to use the claimed invention with a reasonable expectation of success and without undue experimentation" [emphasis added].

The Examiner's Contradictory Positions Regarding "One of Ordinary Skill in the Art"

In the Official Action dated March 19, 2004 (*e.g.*, see page 3, first paragraph through page 6, first paragraph), the Examiner rejected the claims of the invention under 35 U.S.C. § 112, first paragraph (enablement), alleging that Appellants had not provided any convincing evidence that their immunogenic composition is indeed useful for an anti-HIV treatment in humans and had not provided sufficient guidance to allow one skilled in the art to practice the claimed invention with a reasonable expectation of success and without undue experimentation [emphasis added]. No claims were rejected under 35 U.S.C. § 103 in the March 19, 2004 Action.

In a June 18, 2004 Response (page 7, first paragraph page 9, second paragraph), Appellants successfully traversed the enablement rejection, pointing to specific teachings, description and data (*i.e.*, *in vivo* dog and chimpanzee data) in the specification, and further submitting evidence of ongoing HIV Vaccine Trials Network (HVTN) phase I and II studies using HIV env and gag components in immunogenic compositions. The Examiner withdrew the enablement rejection in the subsequent Official Action.

However, in ensuing Actions (dated September 22, 2004, April 21, 2005 and November 11, 2005), the Examiner's arguments/position made a 180° turn with regard to level of knowledge possessed by one skilled in the HIV arts. For example, in the September 22, 2004 Action, the Examiner rejected the pending claims under 35 U.S.C. § 103 over Hung *et al.* or Chanda *et al.*, alleging that: "One of ordinary skill in the art would have been motivated to administer this vaccine to a human to induce an immune response against HIV-1 infection" [emphasis added]. The Examiner further asserted that: "One of ordinary skill in the art at the time the invention was filed would have had a reasonable expectation of success for doing so because Hung *et al.* [and Chanda *et al.*] teaches of vaccines comprising recombinant adenovirus that express HIV1" [emphasis added]. As set forth above, neither Hung *et al.* or Chanda *et al.* teach or described the presently claimed adenovirus vectored prime-boost methods for producing an immune response against HIV-1 infection in a human.

Appellants cannot understand how the Examiner's interpretation of the M.P.E.P., statutes described therein (*i.e.*, 35 U.S.C. § 112 and 103) and case law described therein (discussed below), with respect to the hypothetical "one of skill in the art", can be so diametrically opposed. Furthermore, the Examiner continues to misinterpret the statutes and case law in the Final Office Action dated November 02, 2005. For example, in an unsuccessful attempt to rebut Appellants' "obvious-to-try" arguments, the Examiner states (*e.g.*, see page 6, last paragraph through page 7, second paragraph):

[I]t is noted that Applicant is relying on the state of the art at the time the invention was made to assert that one of ordinary skill in the art would not have been motivated by any of the cited references to administer an Ad-env construct to a human to induce an immune response or would the skilled artisan have had a reasonable expectation of success in doing so based on the teachings provided in the specification, wherein the primary basis for Applicant's assertion in the enablement rejection that was previously issued to Applicant... The factors such as (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) **the state of the prior art**, (6) **the relative skill of those in the art**, (7) **the predictability or unpredictability of the art**, and (8) the breadth of the claims, that are required of an enablement rejection are not required for an obviousness rejection [emphasis added]. The factors that are required for an obviousness rejection are:

- (A) Determining the scope and contents of the prior art;
- (B) Ascertaining the differences between the prior art and the claims in issue;
- (C) Resolving the level of ordinary skill in the pertinent art; and
- (D) Evaluating evidence of secondary considerations.

In the instant case, all of these factors are present in the obviousness rejection issued by the Office. Addition [sic], Applicant's is requested to note that none of these factors includes the state of the art at the time of filing. [emphasis added]

Appellants first wish to point out, that contrary to the Examiner's concluding sentence set forth above, both enablement and obviousness share the nexus of "the level of ordinary skill in the art" (e.g., *Wands* factors (6) and (7) above (see, *In re Wands*, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) and the *Graham* factual inquiries (A)-(C) above (see, *Graham v. John Deere*, 383 U.S. 1, 148 USPQ 459 (1966))). Furthermore, the statute of non-obviousness under 35 U.S.C. § 103 (a) specifically states: "...obvious at the time the invention was made to a person having ordinary skill in the art to which the subject matter pertains." Appellants contend that the Examiner's conclusory statements such as: "... (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability or unpredictability of the art, ... that are required of an enablement rejection are not required for an obviousness rejection", are unsupported.

This point is made abundantly clear in the Examiner's contradictory statements that: (1) there are numerous scientific obstacles related to HIV infection and treatment which "establish that the contemporary knowledge in the art would not allow one skilled in the art to use the claimed invention with a reasonable expectation of success and without undue experimentation" (see, Official Action dated 03/19/2004, page 4, last paragraph), and at the same time, (2) One of ordinary skill in the art would have been motivated to administer this vaccine to a human to induce an immune response against HIV-1 infection" and "had a reasonable expectation of success in doing so because in order to arrive at a administration methodology that would render the most beneficial immune response against HIV-1 infectivity, one of ordinary skill in the art would have necessarily use [sic] various methodology of administering the composition as part of routine experimentation" (see, Final Action dated 11/02/2005, page 3, last paragraph through page 4, first paragraph).

Appellants assert that at the time of the present invention, a person of skill in the art would not have been motivated by the Hung *et al.* reference or the Chanda *et al.* reference to practice the claimed prime-boost methods of administering an Ad-env construct to a human to induce an immune response against HIV-1 infection. Furthermore, in the absence of Appellants' data, one of skill would not have had a reasonable expectation of success based on the disclosure of either the Hung *et al.* reference or the Chanda *et al.* reference. Appellants therefore assert that neither Hung *et al.* nor Chanda *et al.*, taken alone or in combination with Davis *et al.*, render the presently claimed invention obvious. Lastly, Appellants reiterate that they are not claiming the specific Ad constructs described in Hung *et al.* and Chanda *et al.*, rather they are claiming prime boost methods for producing an immune response using similar, but not the same, Ad-env constructs.

Producing an Immune Response Against HIV-1 in a Human

In the Final Action dated November 02, 2005 (page 5, first paragraph) the Examiner sought to explain the obviousness rejection with respect to the teachings of Hung *et al.* and Chanda *et al.*, by differentiating the presently claimed methods for producing an “immune response against HIV-1 infection in a human” versus a hypothetical method for providing “protection against HIV-1 infection”. For example, the Examiner states:

Had it be [*sic*] that the claims are directed to a method of providing protection against HIV-1 infection, the reference(s) that are cited by the Office must necessarily provide an insight on the protective efficacy of the composition or the protective efficacy that can be ascertained of the composition via a unique method of administration. However, this is not instantly present in the claims. Thus, the reference(s) cited by the Office need not to provide any protective efficacy insight.

However, because the claims do require that a production of an immune response against HIV-1 infection, the references must provide an insight on the production of an immune response against HIV-1 infection. In the instant, such insight can be readily deduce from the teachings of the reference(s). The reference(s) teaches that the same vector construct as those cited in the claims having a different antigen, is capable of inducing antibody responses against said antigen. Thus, the a [*sic*] vector construct taught by Hung *et al.* and Chanda *et al.* teach, which is the same as those recited in the claims, would also be capable of inducing an antibody response against said antigen. [emphasis added]

As set forth above, Hung *et al.* and Chanda *et al.* describe a recombinant adenovirus type 7 (Ad7) construct comprising a gene expressing either the hepatitis B surface antigen (HBsAg) or the human immunodeficiency virus type 1 envelope glycoprotein (*env*). Hung *et al.* and Chanda *et al.* demonstrated that intra-tracheal (*i.e.* oral delivery) inoculation of dogs with the Ad7-HBsAg construct induced an antibody response.

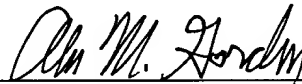
However, neither Hung *et al.* nor Chanda *et al.* performed a similar study with the Ad7-env (*i.e.*, HIV-1) construct, and as such, neither has provided “insight on the production of an immune response against HIV-1 infection”. In fact, there is no *in vitro* or *in vivo* data in either reference to suggest that an Ad7-*env* would in fact elicit an immune in any mammal. Additionally, both references concluded that further research

is needed to fully understand how to generate a significant immune response against HIV-1 infection (*e.g.*, see Hung *et al.*, page 163, Discussion and Chanda *et al.*, page 74, last paragraph through page 76, first paragraph)

Appellants therefore maintain that Hung *et al.* and Chanda *et al.* do not teach or describe the presently claimed prime-boost method for producing an immune response against HIV-1 infection in a human, nor do they provide any "insight on the production of an immune response against HIV-1 infection".

Conclusion

Appellants submit that the pending claims 26 and 28-40 are patentable. It is respectfully requested that the Board reverse the Final Rejection of the subject matter of these claims for the reasons set forth in detail in Section VII.



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VIII. CLAIMS APPENDIX A

- Claim 26. A method for producing an immune response against HIV-1 infection in a human comprising the steps of:
- (1) administering to the human an immunogenic composition comprising an intranasal or an intramuscular dosage of a recombinant adenovirus comprising an expression cassette containing a promoter, a nucleic acid sequence encoding the HIV-1 gp160 or gp120 polypeptide sequence and a polyadenylation signal sequence and
 - (2) administering to the human one or more intranasal or intramuscular booster dosages of the recombinant adenovirus.
- Claim 28. The method of claim 26, wherein the administering one or more booster dosages of the recombinant adenovirus is followed by one or more intramuscular injections of an HIV-1 antigen polypeptide dosage, wherein the antigen polypeptide is a gag polypeptide, an env polypeptide or a combination thereof.
- Claim 29. The method of claim 26, wherein the adenovirus is a serotype 4, a serotype 5 or a serotype 7 adenovirus.
- Claim 30. The method of claim 26, wherein the expression cassette further comprises the coding sequence for the HIV-1 rev gene inserted in frame after the HIV-1 gp160 or gp120 sequence and before the polyadenylation signal sequence.

- Claim 31. The method of claim 26, wherein the HIV-1 gp160 sequence is the MN strain gp160 sequence or the LAV strain gp160 sequence.
- Claim 32. The method of claim 26, wherein the HIV-1 gp160 sequence is replaced by a sequence encoding the gag-pro region of HIV-1.
- Claim 33. The method of claim 26, wherein the intranasal dosage is about 1×10^7 pfu of virus.
- Claim 34. The method of claim 26, wherein the intramuscular dosage is in the range of 1×10^7 to 2×10^9 pfu of virus.
- Claim 35. The method of claim 26, wherein the intranasal booster dosage is in the range of 1×10^7 to 1×10^8 pfu of virus.
- Claim 36. The method of claim 26, wherein the intramuscular booster dosage is in the range of 1×10^{10} to 8×10^{10} pfu of virus.
- Claim 37. The method of claim 28, wherein the antigen polypeptide dosage comprises between 200 μ g and 0.5 mg of antigen polypeptide.
- Claim 38. The method of claim 26, wherein the adenovirus comprises a deletion in the E3 gene.

Claim 39. The method of claim 26, wherein the adenovirus comprises a deletion in the E3 gene and a deletion in the E1 gene.

Claim 40. The method of claim 26, wherein the adenovirus comprises a deletion in the E1 gene.

APPENDIX B

Cited References

- (1) Hung *et al.*, "Adenovirus Vaccine Strains Genetically Engineered to Express HIV-1 or HBV Antigens for Use as Live Recombinant Vaccines", *Nat. Immun. Cell Regul.*, Vol. 9, pages 160-164, 1990 (Attached) , cited by the Examiner at page 4 of the Office Action mailed September 22, 2004.
- (2) Chanda *et al.*, "Helper Independent Recombinant Adenovirus Vectors: Expression of HIV *env* or HBV Surface Antigen", *Intern. Rev. Immunol.*, Vol. 7, pages 66-77, 1990 (Attached) , cited by the Examiner at page 9 of the Office Action mailed September 22, 2004.
- (3) Davis *et al.*, "Oral Vaccines", U.S. Patent 4,920,209, Granted April 24, 1990 (Attached), cited by the Examiner at page 4 of the Office Action mailed September 22, 2004.

APPENDIX C

Related Proceedings Appendix

None.

Adenovirus Vaccine Strains Genetically Engineered to Express HIV-1 or HBV Antigens for Use as Live Recombinant Vaccines

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Key Words. HIV vaccine · HBV vaccine · Recombinant adenovirus vaccine

Abstract. Types 4 and 7 adenovirus are currently used as live, oral vaccines for the prevention of adenovirus respiratory disease in military recruits. These vaccine strains have been genetically engineered in order to express HIV-1 or HBV antigens in infected cells. A dog model was developed to evaluate the immunogenicity of these recombinant vaccines. Dogs inoculated with live adenovirus-HBV recombinant vaccine produced antibody against hepatitis B surface antigen.

Introduction

The oral administration of enterically coated, live, adenovirus vaccines results in an asymptomatic intestinal infection that induces immunity to adenovirus respiratory disease [1, 2]. This history of safe and efficacious use, the ease of oral administration and the extensive literature on adenovirus molecular biology [reviewed in 3] all recommended the use of adenovirus as a live recombinant vaccine/vector. We have reported a number of studies that illustrate our progress in the development of recombinant adenovirus vectors that express hepatitis B surface antigen (HBsAg) [4-6] or the HIV-1 envelope glycoprotein (ENV) [7] in

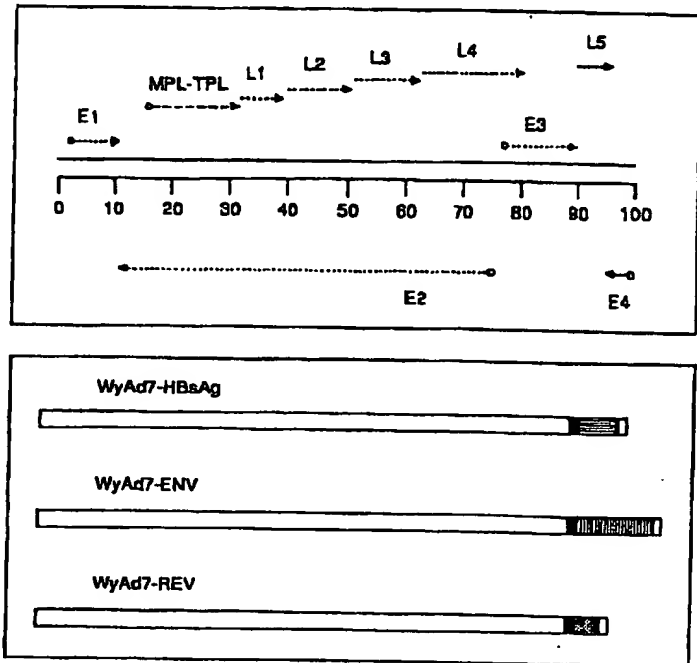
infected cells. In this review, we will focus on the factors affecting the level of expression of HIV-1 ENV in adenovirus expression systems, and we report the use of dogs as a model for adenovirus type 7 (Ad7) infection.

Results

Construction of Recombinant Adenoviruses

The recombinant adenoviruses described in figure 1 were constructed using techniques previously described [4]. In summary, a genetic cassette containing a heterologous viral antigen gene under the transcrip-

Fig. 1. Structure of recombinant adenoviruses. **a** Simplified diagram of the transcript map of Ad7, each MU is approximately 360 base pairs. MLP = Major late promoter; TPL = tripartite leader. **b** Recombinant adenoviruses described here all contain a genetic expression cassette inserted between the E4 region and the right inverted terminal repeat that consists of a duplicate MLP and TPL (■), a heterologous viral gene from which the endogenous promoter has been edited, and a duplicate adenovirus polyadenylation signal (black bar). The genetic cassette of WyAd7-HBsAg contains the HBsAg gene (■), that of WyAd7-ENV contains the HIV-1 ENV gene (■) and that of WyAd7-REV contains the HIV-1 REV gene (■). These recombinant adenoviruses all have deletions in the E3 region as noted in the text.



tional control of the adenovirus major late promoter and the translational control of the adenovirus tripartite leader was inserted into a cloned fragment of adenovirus DNA. The recombinant fragment, extending from MU 70 to MU 100, was transfected together with a genomic fragment of Ad7 DNA extending from MU 0 to MU 87 and infectious recombinant virus was generated by homologous recombination.

The genetic cassette employed in these studies differs from earlier cassettes in that most of the intervening sequence between the first and second parts of the tripartite leader is retained. This resulted in significantly enhanced expression, as will be described in detail elsewhere.

In order to accommodate the genetic cassettes within the adenovirus packaging limit, these recombinant adenoviruses were constructed with a large deletion (approximately 7 MU) in the nonessential E3 region.

Production of HBsAg and ENV in Cells Infected with Recombinant Adenoviruses

As no essential genes were removed in the construction of these recombinant adenoviruses, they can be propagated in ordinary human cell lines, such as A549, in the absence of helper virus. Cells infected with WyAd7-HBsAg yield a total of approximately 7 μ g of HBsAg/ 10^6 cells, assayed by RIA (AUSRIA II, Abbott). This level of production is similar to that of some of the ade-

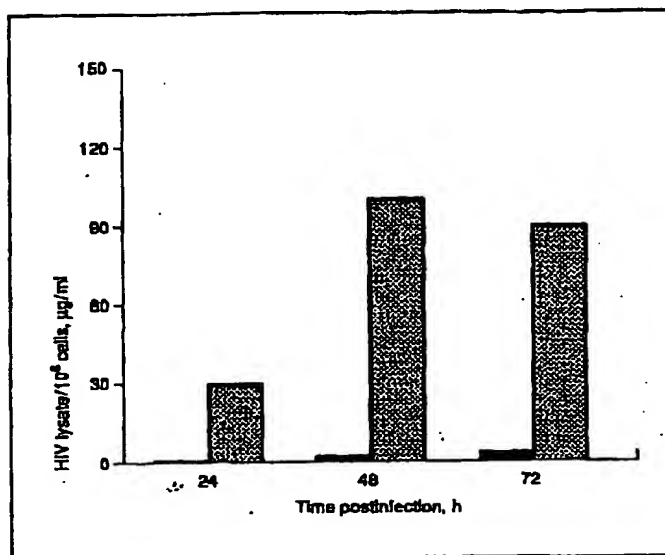


Fig. 2. Expression of HIV-1 ENV. Cells were either infected with WyAd7-ENV (■) or coinfecting with both WyAd7-ENV and WyAd7-REV (▨) and then extracted with RIPA buffer at the indicated intervals after infection. The level of ENV in these lysates was assayed by ELISA as described in the text. A heat-inactivated viral lysate of HIV-1 was used as a standard; the amount of ENV produced/10⁶ cells infected with recombinant adenovirus is reported as the equivalent concentration of heat-inactivated viral lysate (measured as µg total protein/ml). Please note that the heat-inactivated HIV viral lysate contains many proteins in addition to ENV; ENV is only a small fraction of the total protein.

novirus structural proteins, and HBsAg is a prominent band in the SDS-PAGE pattern of a metabolically radiolabelled, virus-infected, cell lysate [data not shown]. Although difficult to quantitate, the yield of ENV from cells infected with WyAd7-ENV seemed to be at least 10-fold less than the yield of HBsAg from cells infected with WyAd7-HBsAg; ENV could be observed by SDS-PAGE only after immunoprecipitation. These results suggested that although the ENV gene was under the same transcriptional control as the HBsAg gene, some post-transcriptional block to ENV expression was operating in the adenovirus-infected cell.

Since the HIV-1 TAT and REV gene products had been shown to enhance ENV expression from transient expression vectors that use the HIV-1 LTR as promoter [8, 9], we constructed WyAd7-REV. Figure 2 illustrates the enhanced expression of ENV ob-

served when cells were coinfecting with WyAd7-ENV and WyAd7-REV. ENV expression was assayed by using a modified HIV antigen capture ELISA (Cellular Products) in which a biotinylated mouse monoclonal antibody specific for ENV (Pan Data Systems) was substituted for the original anti-HIVp24 monoclonal antibodies. The REV gene product increased the yield of ENV by as much as 50-fold so that ENV was visible as a prominent band upon SDS-PAGE analysis of a metabolically radiolabelled coinfecting cell lysate [data not shown]. Preliminary S-1 analysis of ENV-specific mRNA from cells either infected with WyAd7-ENV or coinfecting with both WyAd7-ENV and WyAd7-REV indicates only a 2- to 3-fold increase in steady-state mRNA levels, suggesting an additional post-transcriptional effect of REV on ENV expression [data not shown].

Table 1. Anti-HBsAg response of dogs inoculated with WyAd7-HBsAg

Dose, PFU	Anti-HBsAg response pos./inoc. ¹			
	0w	2w	4w	8w
10 ⁹	0/4	4/4	3/4	3/4
UV(10 ⁹) ²	0/2	0/2	0/2	0/2

pos. = Positive; inoc. = inoculum; w = week; UV = ultraviolet.

¹ Negative responses were ≤ 20 mIU; the geometric mean titer of each group of positive responses was ≥ 200 mIU.

² Inocula corresponding to 10⁹ PFU/dose were irradiated with ultraviolet radiation to reduce infectivity to approximately 10² PFU/dose.

Immunogenicity of WyAd7-HBsAg in Dogs

We have previously reported the use of hamsters as a small animal model for adenovirus type 5 (Ad5) infection [5, 10]. Because hamsters do not support the replication of Ad7, we investigated the replication of Ad7 in dogs. Table 1 demonstrates that the intratracheal inoculation of dogs with WyAd7-HBsAg induced an antibody response to HBsAg, as measured by RIA (AUSAB, Abbott). Inactivation of the inoculum by ultraviolet irradiation prevented the production of anti-HBsAg, indicating that viral replication is necessary to induce the immune response.

Discussion

These results review recent progress in our development of the vaccine strains of adenovirus as live recombinant vaccines for hepatitis B and AIDS. The demonstration of

enhanced ENV expression in the presence of REV is remarkable because previous studies [8, 9] using the HIV-1 LTR as the promoter were necessarily done in the presence of TAT. A recent study in which a proviral fragment of the HIV-1 genome that contains the TAT, REV and ENV open reading frames was inserted behind an SV40 promoter demonstrated the synthesis of REV and ENV but did not exclude the possibility that TAT was synthesized, as well [11]. Our study clearly demonstrates that REV can enhance ENV production in the absence of TAT. In control experiments, coinfection of cells with WyAd7-HBsAg and WyAd7-REV did not enhance HBsAg production. We are investigating this effect in more detail.

One way to increase the immunogenicity of our recombinant adenoviruses is to boost antigen expression levels; a parallel path is to increase the immunogenicity of the antigen that is presented. Recombinant adenoviruses that contain the HIV-1 GAG-POL genes have also been constructed and the expression of these HIV-1 antigens is under study. The ultimate recombinant adenovirus vaccine for AIDS may be designed for the expression of several antigens simultaneously. The antigens might also be genetically engineered to enhance their immunogenicity [12]. Use of the dog model should permit rapid evaluation of immunogenicity and facilitate the decision of which recombinant adenoviruses will be further tested in primates.

Acknowledgments

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References

- 1 Couch, R.B.; Chanock, R.M.; Cate, T.R.; Lang, D.J.; Knight, V.; Huebner, R.J.: Immunization with types 4 and 7 adenovirus by selective infection of the intestinal tract. *Am. Rev. resp. Dis.* 88: 394-403 (1963).
- 2 Top, F.H., Jr.; Buescher, E.L.; Bancroft, W.H.; Russell, P.K.: Immunization with live types 7 and 4 adenovirus vaccines. II. Antibody response and protective effect against acute respiratory disease due to adenovirus type 7. *J. infect. Dis.* 124: 155-160 (1971).
- 3 Ginsberg, H.S. (ed): *The adenoviruses* (Plenum Press, New York 1984).
- 4 Davis, A.R.; Kostek, B.; Mason, B.B.; Hsiao, C.L.; Morin, J.; Dheer, S.K.; Hung, P.P.: Expression of hepatitis B surface antigen with a recombinant adenovirus. *Proc. natn. Acad. Sci. USA* 82: 7560-7564 (1985).
- 5 Morin, J.E.; Lubeck, M.D.; Barton, J.E.; Conley, A.J.; Davis, A.R.; Hung, P.P.: Recombinant adenovirus induces antibody response to hepatitis B virus surface antigen in hamsters. *Proc. natn. Acad. Sci. USA* 84: 4626-4630 (1987).
- 6 Molnar-Kimber, K.L.; Jarocki-Witek, V.; Dheer, S.K.; Vernon, S.K.; Conley, A.J.; Davis, A.R.; Hung, P.P.: Distinctive properties of the hepatitis B virus envelope proteins. *J. Virol.* 62: 407-416 (1988).
- 7 Hung, P.P.; Morin, J.E.; Lubeck, M.D.; Barton, J.E.; Molnar-Kimber, K.L.; Mason, B.B.; Dheer, S.K.; Jarocki-Witek, V.; Kostek, B.; Zandle, G.; Conley, A.J.; Davis, A.R.: Expression of HBV surface antigen or HIV envelope protein using recombinant adenovirus vectors. *Nat. Immun. Cell Growth Regul.* 7: 135-143 (1988).
- 8 Knight, D.M.; Flomerfelt, F.A.; Ghayech, J.: Expression of the art/tra protein of HIV and study of its role in viral envelope synthesis. *Science* 236: 837-840 (1987).
- 9 Sodroski, J.; Goh, W.C.; Rosen, C.; Dayton, A.; Terwilliger, E.; Haseltine, W.: A second post-transcriptional trans-activator gene required for HTLV-III replication. *Nature, Lond.* 321: 412-417 (1986).
- 10 Molnar-Kimber, K.L.; Davis, A.R.; Jarocki-Witek, V.; Lubeck, M.D.; Vernon, S.K.; Conley, A.J.; Hung, P.P.: Characterization and assembly of hepatitis B envelope proteins expressed by recombinant adenovirus. In Robinson, Koike, Will, Hepadna virus, pp. 173-187 (Liss, New York 1987).
- 11 Rekosh, D.; Nygren, A.; Flodby, P.; Hammarshjold, M.-L.; Wigzell, H.: Co-expression of human immunodeficiency virus envelope proteins and tat from a single simian virus 40 late replacement vector. *Proc. natn. Acad. Sci. USA* 85: 334-338 (1988).
- 12 Millich, D.R.; Hughes, J.L.; McLachlan, A.; Thornton, G.B.; Moriarty, A.: Hepatitis B synthetic immunogen comprised of nucleocapsid t-cell sites and an envelope B-cell epitope. *Proc. natn. Acad. Sci. USA* 85: 1610-1614 (1988).

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Helper Independent Recombinant Adenovirus Vectors: Expression of HIV *env* or HBV Surface Antigen

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KEYWORDS: *recombinant viral vaccines, recombinant adenovirus vectors, adenovirus vectors in AIDS vaccines*

INTRODUCTION

Live adenovirus vaccines were originally developed to control the acute respiratory disease observed in military recruits quartered in barracks [1]. Live adenovirus vaccines, types 4 and 7 are currently in use. They are administered as enteric-coated tablets that dissolve in the intestine where the virus replicates asymptotically giving rise to immunity against respiratory disease. The history of safe and efficacious use, the ease of oral administrations and the extensive literature available on adenovirus molecular biology prompted us to use adenovirus as a live recombinant vaccine vector. We have previously reported a number of studies which illustrate the use of recombinant adenovirus to express hepatitis B surface antigen (HBsAg) [2–4]. In this article, we have chosen adenovirus vector to express HIV-1 *env* glycoprotein gene products. It has been reported from this laboratory that type 4 and type 7 adenoviruses have the ability to replicate in the intestinal tract of chimpanzee, one of the few animal models available for HIV-1 infection and replication. Recombinant adenovirus containing HIV *env* were constructed to explore their potential use as live vector vaccines against AIDS. Moreover the asymptomatic infection by recombinant adenovirus may provide protection against AIDS by inducing a broader spectrum of immune responses including cellular, humoral and mucosal immunity against HIV-1 *env*.

STRATEGIES FOR CONSTRUCTION OF RECOMBINANT ADENOVIRUS CARRYING FOREIGN GENES

1. Insertion of Genes in the Region of Adenovirus Genome Essential for Viral Replication

The use of adenovirus as a vector by which foreign genes can be inserted under the viral regulatory control provides an attractive system for introducing genes into mammalian cells.

Foreign DNA sequences can be inserted either in the essential or non-essential regions of adenovirus genome for replication. When extensive substitution replaces sequences required for permissive infection, then the resulting defective virus must be grown with a complementing helper under selective conditions that maintain the defective virus in the population. Such type of adeno-SV40 recombinants were made in which SV40 early region was placed under the control of either an ecotropic copy of the adenovirus major late promoter [5] or several of the conventional promoters [6]. Another type of recombinant adenoviruses with deletion in E1 region, however, can be grown in transformed cell lines [7] without the helper virus. Usually, these transformed cell lines provide the constitutive expression of the E1 protein. Examples of such recombinant adenoviruses that expressed hepatitis B virus surface antigen (HBsAg) have been documented [2, 4, 8]. Although such type of recombinant adenoviruses might be useful for expression of foreign genes, they have limited potential for use as live vaccine candidates. This is because they are unable to replicate independently *in vivo*.

2. Insertion of Genes in Regions of Adenovirus Genome Non-essential for Viral Replication

The proteins of the adenovirus E3 region are well conserved among different subgroups. Moreover, they have been shown to contribute persistence infection by masking the infected cells from immune surveillance mechanism [9, 10] and also from the antiviral effect of tumor necrosis factor [11]. The E3 region has also been shown not to be essential for viral replication in tissue culture [12, 13] and in animal model [3, 14]. Thus, E3 region seems to be a likely site for insertion of foreign gene. Morin *et al.* [3] have demonstrated the expression of HBsAg from E3 region using Ad5 serotype. They have also shown that this recombinant adenovirus can induce antibody response to HBsAg in hamster, another suitable animal model for Ad5 replication. Thus the dispensable E3 region coupled with the ability of adenovirus to package sequences up to 105 map units, allow for considerable substitution.

Besides E3 regions, there are, however, regions of the genome that are not transcriptionally active. Such region may also serve as an alternate insertion site for foreign gene. A recombinant adenovirus that carries the hepatitis B virus genome inserted in the region between E4 and right-hand ITR at map unit 100 has been constructed [15]. Insertion of gene-like HBsAg behind either E1 [8] or E3 promoter [3] can produce a significant amount of HBsAg only during the early phase of infection. However, when the infection proceeds during the late phase, these early promoters become less active. At late phase, most of the mRNA species are derived from the major late promoter (MLP) as the viral genome copy number increases thus establishing a post-transcriptional regulation of gene expression. The transcriptional map pattern of adenovirus mRNA is shown in Figure 1. In order to exploit these effects, an expression cassette was constructed that can be easily transposed as an independent transcription unit. This expression cassette contains cloned copy of MLP, followed by tripartite leader and polyadenylation signal. The adenovirus tripartite leader was included to enhance the translation of late mRNA containing tripartite leader. The effect of tripartite leader on the enhancement of several proteins including adenovirus E1 protein [16], mouse dehydrofolate reductase [17], and HBsAg [2] have been reported when the corresponding mRNA includes tripartite leader. Different polyadenylation signals including SV40, hepatitis B virus, adenovirus early and late transcription regions have been successfully utilized in different versions of the expression cassette. Additional sequence elements such as a putative adenovirus enhancer region [18] and RNA splicing signals have been included to enhance the expression.

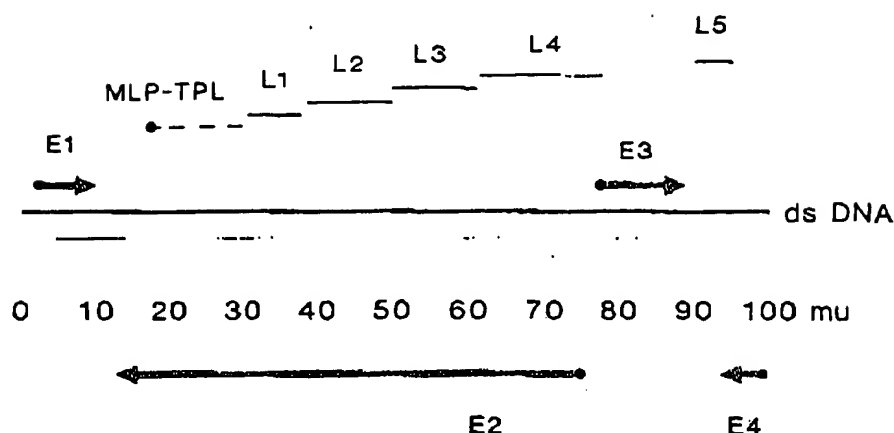


FIGURE 1 Transcriptional organization of the Ad2 genome. For reference purposes, the genome is divided into 100 map units (m.u.); each m.u. is ~360 bp long. Closed circles indicate the positions of transcriptional promoters. Striped lines with arrows show early transcription regions (E1, E2, E3, and E4); solid lines with arrow depict the family of late RNAs (L1, L2, L3, L4, and L5) that is derived from the alternate RNA splicing from major late transcription region. The arrowheads represent the 3' polyadenylation sites.

DESIGN FOR CONSTRUCTION OF RECOMBINANT ADENOVIRUS EXPRESSION VECTOR

Because of the large size (~35Kb) of the adenovirus genome and the difficulties associated with manipulation of such size of DNA, the first step towards construction of recombinant expression vectors involves the cloning of a portion of adenovirus genome for the insertion of heterologous DNA [11, 19]. The sequence of that portion of the adenovirus genome should preferably be known in order to identify different transcription units within that region. Heterologous DNA can be inserted either as a part of the expression cassette under the control of the MLP located in the region like between E4 and right-hand ITR at 100 map unit or under the control of an endogenous adenovirus promoter, like the E3 promoter. This cloned recombinant adenovirus can be used to create a recombinant adenovirus by homologous recombination *in vivo* by transfecting the linearized form of this DNA in cultured cells along with a purified adenovirus DNA fragment that overlaps the cloned adenovirus sequence so that the transfected fragments together include the entire genome.

CONSTRUCTION AND EXPRESSION OF RECOMBINANT ADENOVIRUSES CARRYING HIV-1 *env* GENE

The entire coding sequence (gp160) of the envelope gene of the human immunodeficiency virus HIV-1 [20], was inserted into an expression cassette which contained Ad7 MLP, tripartite leader and an intron in between leader 1 and leader 2 followed by adenovirus hexon polyadenylation sequence. The expression cassette containing the HIV-1 *env* gene was then inserted at the right-hand end of the viral genome between the E4 promoter region and the inverted terminal repeat at a position 159 bp from the extreme right terminus of the Ad7 genome. Additionally this recombinant adenovirus contained an 80–87 m.u. E3 deletion. Recombinant adenovirus, Ad7-*env*, was obtained by homologous recombination *in vivo* (Fig. 2).

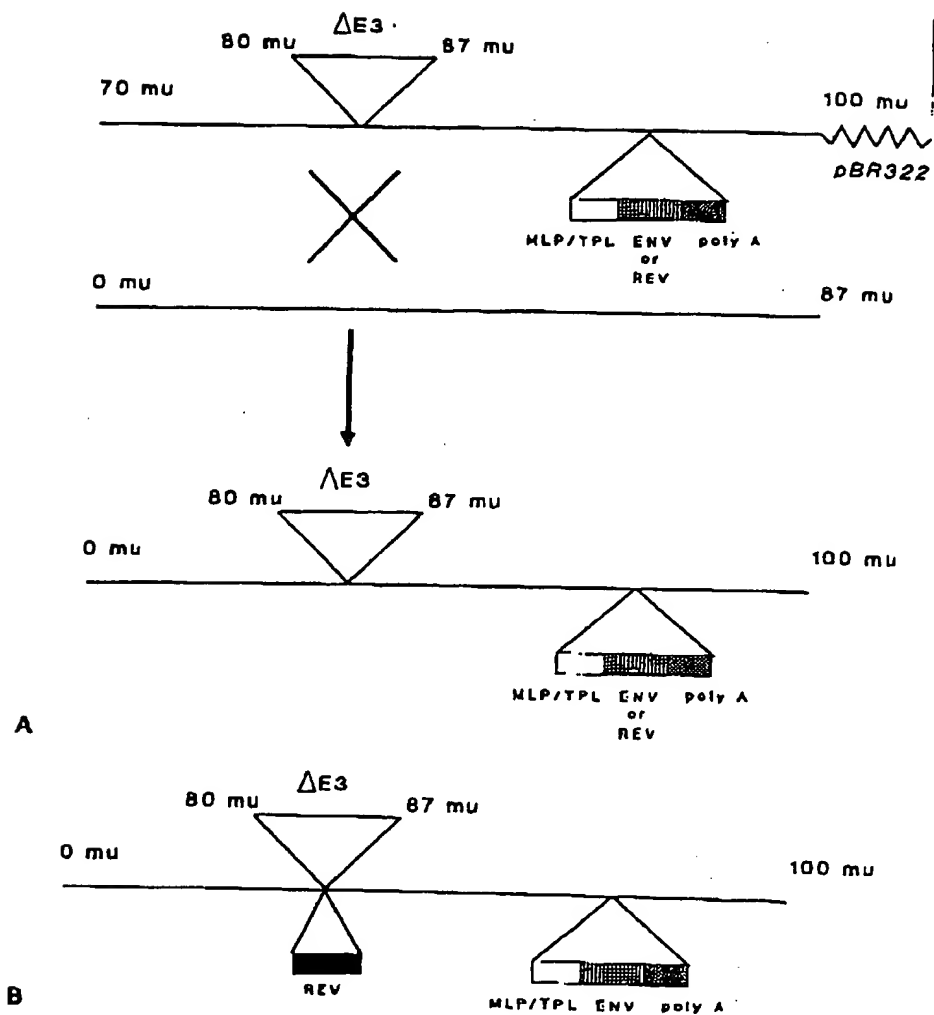


FIGURE 2 A: Construction of Ad7-*env*, Ad7-*rev* viruses. A 2.56 kb DNA fragment containing the HIV-1 *env* gene (gp160) [20] coding sequence or 360 bp DNA fragment coding for the *rev* gene was inserted in the expression cassette. The expression cassette contains major late promoter (MLP), tripartite leader (TPL) with an intervening sequence between leader 1 and leader 2, and hexon polyadenylation sequence. The expression cassette containing the *env* or *rev* genes was inserted at the XbaI site created at 159bp from the right-hand end of the viral genome in between the E4 promoter and the ITR region. The final plasmid for *in vivo* recombination contained, in addition, m.u. 70 to 100 and a deletion in the E3 region between m.u. 80-87. B: Construction of Ad7-*rev-env* virus. This recombinant virus contains both *rev* and *env* genes coding sequences. *Rev* gene was inserted in the deleted (80-87 m.u.) E3 region while the *env* gene was placed in the terminal cassette as described in A. Recombinant adenoviruses were obtained via homologous recombination *in vivo* using calcium phosphate-glycerol method [12].

EFFECT OF THE *rev* GENE ON HIV-1 *env* SYNTHESIS

Since the *rev* gene has been shown to enhance the expression of HIV-1 *env* protein [21-23], we wanted to investigate the effect of *rev* gene on HIV-1 *env* synthesis in our recombinant Ad7 expression system. For this purpose, recombinant adenovirus containing *rev* gene was constructed the same way that the *env* gene was constructed (Fig. 2) except that the *env* sequence was replaced by synthetic *rev* sequence. This *adeno-rev* recombinant, Ad7-*rev* will provide *rev* gene function at late times after infection from the major late promoter. This

recombinant virus produced the *rev* gene product, a 19-kd protein [24-27] that can be demonstrated by Western blotting (Fig. 3) using rabbit anti-*rev* serum. The effect of *rev* gene on HIV-1 *env* synthesis was then investigated by co-infecting A549 cells with both Ad7-*env* and Ad7-*rev* recombinants. Following infection, production of *env* glycoproteins were monitored by analyzing radiolabeled extracts from infected cells. As shown in Figure 4, expression of *env* protein was enhanced at least 20- to 50-fold in the presence of *rev* gene. However, the steady state cytoplasmic accumulation of *env*-specific mRNAs after the co-infection of Ad7-*env* and Ad7-*rev* were only three- to fivefold greater as compared with Ad7-*env* recombinant. This indicates that the *rev*-mediated post-transcriptional regulation of *env*-expression that has been established in HIV-1 proviral system also plays a role in adenoviral expression vectors. This type of *rev*-mediated *env* gene enhancement has not been reported in vaccinia virus expression system wherein DNA replication occurs in cytoplasm.

CO-EXPRESSION OF *rev* AND *env* GENES FROM SINGLE RECOMBINANT ADENOVIRUS

Since *rev* can enhance HIV-1 *env* production at least 20- to 50-fold, we wanted to construct an adenovirus type 7 recombinant that can express both *rev* and *env* genes from the same virus. For this purpose, *rev* gene was inserted in the E3 region under the control of E3

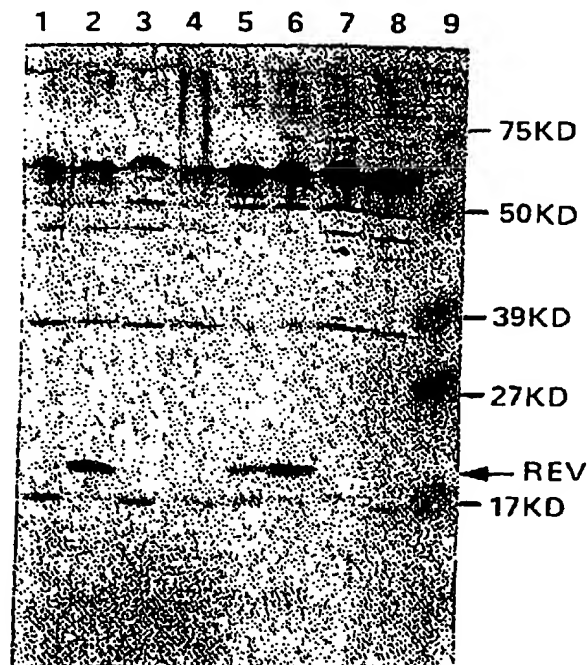


FIGURE 3 Western blot analysis of *rev* protein produced by Ad7-*rev* and Ad7-*rev-env* viruses in HeLa or HeLa T4⁺ cells. HeLa or HeLa T4⁺ cells were infected by Ad7-*rev*, Ad7-*rev-env* as well as wild-type Ad7 viruses. Lysates were prepared [2, 34], electrophoresed on 15% SDS-polyacrylamide gel [35] and the *rev* protein was detected by immunoblotting using rabbit antiserum raised against *rev-tpE* fusion protein. Lanes: 1, Ad7-*rev-env*; 2, Ad7-*rev*; 3, wild-type Ad7; 4, HeLa cells; 5, Ad7-*rev-env*; 6, Ad7-*rev*; 7, wild-type Ad7; 8, HeLa T4⁺ cells; 9, pre-stained protein molecular weight standards (Bio-Rad).

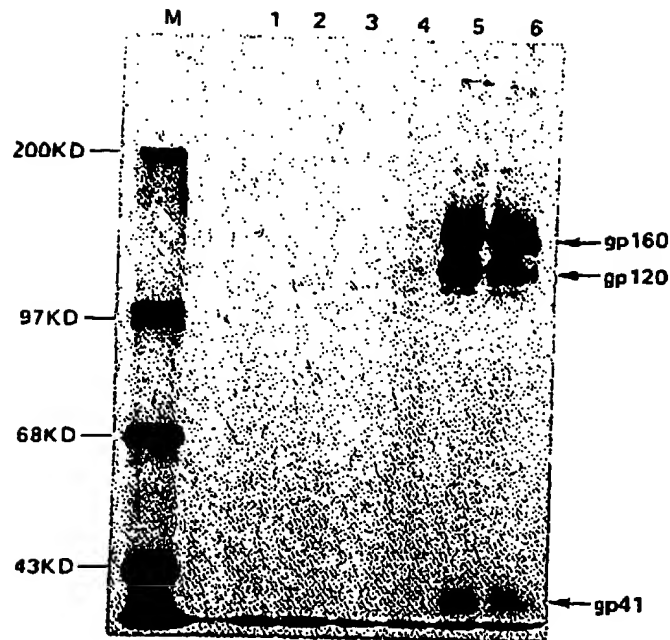


FIGURE 4 Electrophoretic analysis of HIV-1 *env* proteins produced in A549 cells by recombinant adenoviruses. Infected A549 cells were metabolically radiolabeled with (35 S) cysteine, and the radiolabeled extracts were prepared using RIPA buffer [2, 34]. The HIV-1 *env* proteins were immunoprecipitated using the serum from the AIDS patient known to contain high titer antibodies against HIV-1 *env* proteins. Lanes: 1, A549 cells; 2, wild-type Ad7; 3, Ad7-*rev*; 4, Ad7-*env*; 5, Ad7-*env* and Ad7-*rev* viruses (Ad7-*env* + Ad7-*rev*); 6, Ad7-*rev-env*. The size and location of protein markers are indicated.

promoter with an E3 deletion from 80–87 m.u. while the *env* gene was inserted in the terminal cassette resulting in the recombinant adenovirus Ad7-*rev-env* (Fig. 2). This recombinant adenovirus will produce *rev* gene at early times after infection, while the *env* protein will be produced at late times after infection. This type of approach is appealing as two different genes can be simultaneously expressed from two different promoters and the gene products can interact functionally, e.g., one from the MLP and the other from the E3 promoter. RNA analysis of *rev* gene in E3 region from Ad7-*rev-env* indicated authentic E3 promoter-driven *rev* mRNA that end at E3B polyadenylation site. This *rev* mRNA of early promoter would yield only low quantities of *rev* protein during the early stages of infection. However, during late stages of infection, the *rev* gene is embedded in the late L4 readthrough mRNAs and becomes polycistronic type. Hence, the translation efficiency of *rev* gene is less probable during late times even though the *env* gene in MLP cassette is expressed mainly at late stages. The result of the expression of the *env* protein by this recombinant is shown in Figure 4. This recombinant produced similar levels of *env* as compared to the double infections with both Ad7-*env* and Ad7-*rev* viruses indicating the stability or requirement for only a small amount of *rev*.

CONSTRUCTION OF AN ALTERED *rev* GENE TO ELIMINATE SEQUENCE DUPLICATION IN Ad7-*rev-env* BY *rev*

To eliminate sequence duplication in Ad7-*rev-env* that arises from the second exon of *rev* gene, an altered *rev* gene was chemically synthesized with the help of codons that are

frequently used in human genes [28]. This was done to avoid genetic instability that might be caused due to duplicated sequences. This *rev* gene, *Hrev*, was expressed from the terminal cassette and was found to be functional in terms of its ability to enhance *env* synthesis compared to the unaltered *rev* gene (Fig. 5). This experiment strongly suggests that the *rev* protein p19 and not the RNA is responsible for its interaction with the *rev* responsive element, RRE.

CONSTRUCTION OF OTHER POTENTIAL VACCINE CANDIDATES

Some of the other potential vaccine candidates were constructed either by placing the cassette at different locations to improve growth or by elimination of IVa2 promoter to avoid potential interference from the MLP transcription in the cassette (data not shown). Also some of the constructs contained larger E3 deletion to minimize overpackaging which might cause genetic instability.

FUNCTIONAL ANALYSIS OF HIV-1 *env* PROTEIN PRODUCED BY RECOMBINANT ADENOVIRUS Ad7-*rev-env*

To investigate whether the *env* protein produced by recombinant adenovirus, Ad7-*rev-env*, was functional or not, the formation of syncytia was monitored in HeLa T4⁺ cells (Fig. 6). Syncytia could only be detected from the recombinant Ad7-*rev-env* expressing high levels of *env* protein but not from the uninfected cell cultures or cell cultures infected with wild-type Ad7 virus. Since syncytia formation involves the interaction between CD4 molecule and the *env* glycoproteins expressed on the cell surface [29–31], the experiment suggests that some *env* protein was expressed on the surface of HeLa T4⁺ cells.

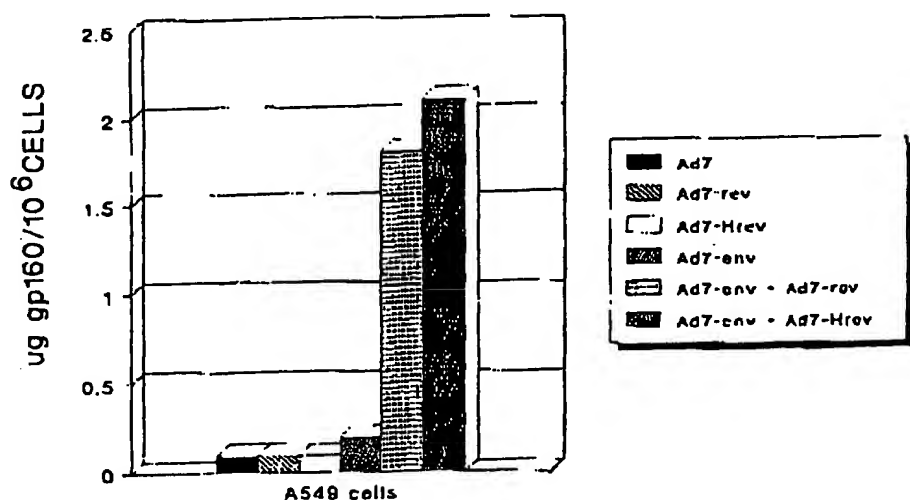


FIGURE 5 Analysis of HIV-1 *env* protein in presence of *Hrev*. A549 cells were infected with various recombinant adenoviruses as shown in the box and the whole cell extracts were analyzed by *env*-ELISA using a commercial HIV-1 p24 antigen capture assay (Cellular Products Inc., Buffalo, NY) that was modified to detect HIV-1 gp160/*env* expression.

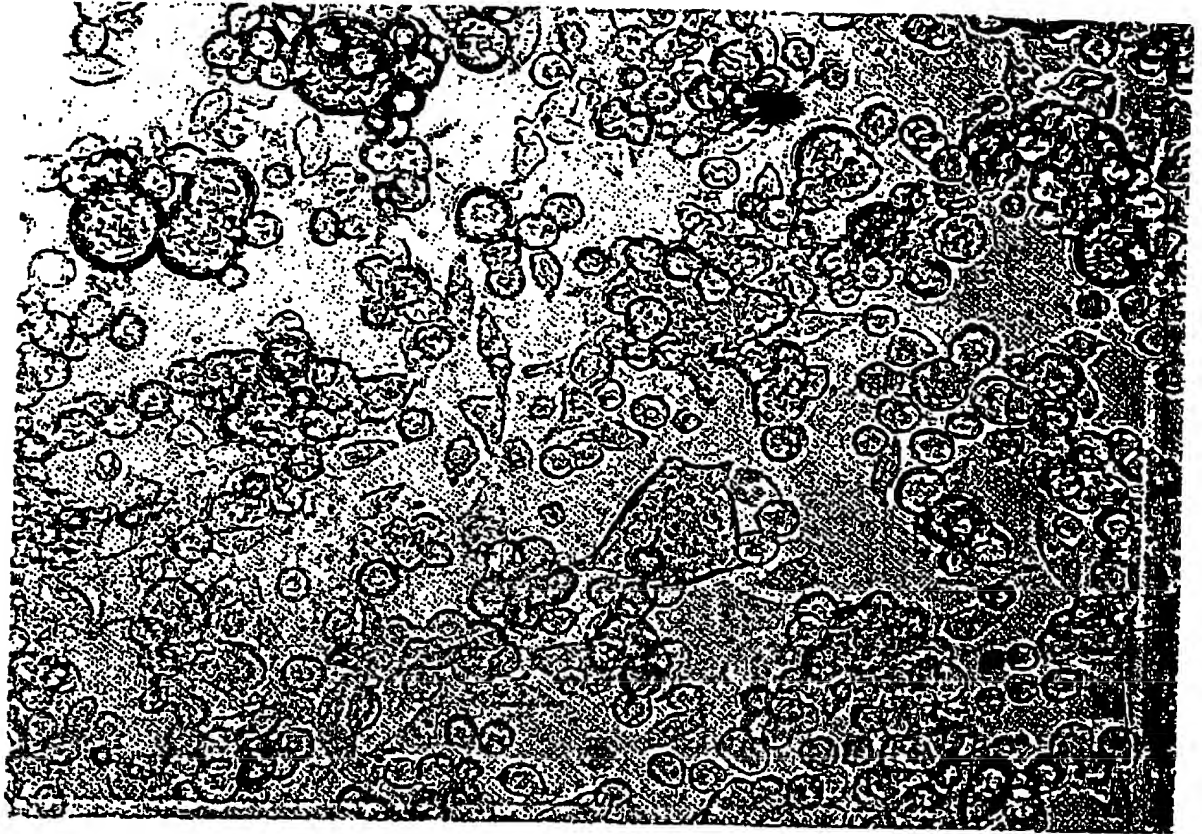


FIGURE 6 Syncytia formation by Ad7-*rev-env* virus. HeLa T4⁺ cells were infected with Ad7-*rev-env*, and the syncytia were observed beginning 40–60 h post infection.

EVALUATION OF ADENO-HIV CONSTRUCTS IN TISSUE CULTURE

The constructs were evaluated in A549 cell lines [32] and in low passaged HEK cells [33]. The result is shown in Figure 7. As can be seen from Figure 7, similar amounts of *env* protein was produced in both types of cells.

IMMUNIZATION OF EXPERIMENTAL ANIMALS WITH RECOMBINANT ADENOHEPATITIS VIRUS

Human Ad4 and Ad7 exhibit a highly restricted host range and replicate poorly in all experimental animals except the chimpanzee. Screening of numerous non-primate species for permissivity to adenovirus infections identified the dog as exhibiting the highest and most consistent humoral immune responses to Ad7-vectored vaccines. However, the dog is only semi-permissive for Ad7 replication and requires intratracheal administration of high doses of virus to induce immune responses. Table I represents seroresponses of dogs to the Ad7-hepatitis B recombinant virus WyAd7IHH-1. This virus contains the HBsAg coding sequence inserted in the right-hand terminus of the Ad7 genome in the context of an expression cassette. Dogs immunized at doses of 10^9 pfu exhibited significant anti-HBs

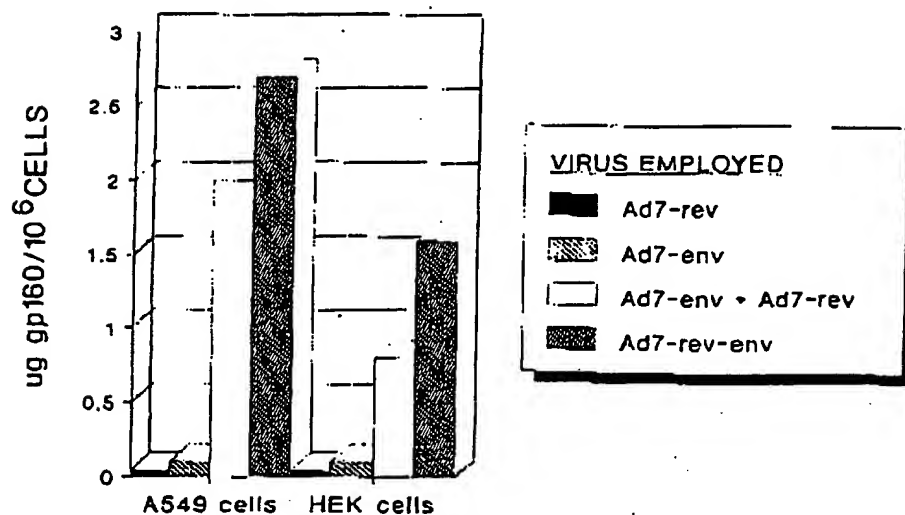


FIGURE 7 Expression of HIV-1 *env* in A549 and HEK cells. Cells were infected with recombinant adenoviruses as indicated in the box. Whole cell extracts were prepared, and the HIV-1 *env* proteins were measured by env-ELISA.

responses, whereas immunization of dogs at lower doses resulted in inconsistent seroconversion to HBsAg. These data demonstrate that under semi-permissive conditions, adenovirus vectors may induce seroconversion to products of foreign gene inserts.

DISCUSSION

Recombinant adenovirus has been widely used to study gene expression. However, the majority of these vectors are unable to replicate independently *in vivo*. We have constructed recombinant adenoviruses derived from the currently used live, oral adenovirus vaccine strain 7, that can express high levels of HIV-1 *env* glycoproteins in presence of *rev* gene and can grow in a helper-independent fashion. The *env* glycoprotein produced by those recombinant adenoviruses was found to be biologically active. Moreover, under semi-permissive

TABLE I
Seroresponses of Dogs to WyAd7IHH-1

Doses (pfu)	Number of dogs per group	Anti-Ad7 [†]		Anti-HBs [‡] (no. positive/no. tested)	
		pre	4w	pre	4w
10 ⁹	4	10	>640	0/4	4/4
10 ⁹ —U.V.	2	<10	<10	0/4	0/2
10 ⁸	4	<10	640	0/4	1/4
10 ⁷	4	<10	160	0/4	0/4
10 ⁶	4	<10	40	0/4	2/4

[†]Geometric mean titers.

[‡]Positive responses ≥ 10 mIU.

conditions, recombinant adenovirus vector type 7 may induce seroconversion to hepatitis B virus surface antigen in dogs which can be utilized as an animal model for evaluating the constructs as vaccine candidates. Recently, protective vaccine against hepatitis B virus using recombinant adenovirus has been demonstrated in chimpanzees [14]. Further animal studies, using chimpanzee with the adeno-HIV-1 constructs, will better define the full spectrum of immune responses to HIV-1 *env* protein. Such studies are currently in progress.

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References

1. Couch, R. B., Chanock, R. M., Cate, T. R., Lang, D. J., Knight, V., and Huebner, R. J. Immunization with types 4 and 7 adenovirus by selective infection of the intestinal tract. *Amer. Rev. Resp. Dis.* 88, 394, 1963.
2. Davis, A. R., Kostek, B., Mason, B., Hsiao, C. L., Morin, J., Dheer, S. K., and Hung, P. P. Expression of hepatitis B surface antigen with a recombinant adenovirus. *Proc. Natl. Acad. Sci. USA* 82, 7560, 1985.
3. Morin, J. E., Lubeck, M. D., Barton, J. E., Conley, A. J., Davis, A. R., and Hung, P. P. Recombinant adenovirus induces antibody response to hepatitis B virus surface antigen in hamsters. *Proc. Natl. Acad. Sci. USA* 84, 4626, 1987.
4. Molnar-Kimber, K. L., Jarocki-Witek, V., Dheer, S. K., Vernon, S. K., Conley, A. J., Davis, A. R., and Hung, P. P. Distinctive properties of the hepatitis B virus envelope proteins. *J. Virol.* 62, 407, 1988.
5. Solnick, D. Construction of an adenovirus-SV40 recombinant producing SV40 T antigen from an adenovirus late promoter. *Cell* 24, 135, 1981.
6. Thummel, C., Tjian, R., and Grodzicker, T. Expression of SV40 T antigen under control of adenovirus promoter. *Cell* 23, 825, 1981.
7. Graham, F. L., Smiley, J., Russell, W. C., and Nairn, R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* 36, 59, 1977.
8. Balley, A., Leviero, M., Ovendia, M. A., Tiollais, P., and Porricaudet, M. *In vitro* and *in vivo* synthesis of the hepatitis B virus surface antigen and of the receptor for polymerized human serum albumin from recombinant human adenoviruses. *EMBO J.* 4, 3861, 1985.
9. Burgert, H. G. and Kvist, G. An adenovirus type 2 glycoprotein blocks cell surface expression of human histocompatibility class I antigen. *Cell* 41, 987, 1985.
10. Seversinsson, L. and Paterson, P. A. Abrogation of cell surface expression of human class I transplantation antigens by an adenovirus protein in *Xenopus laevis* oocytes. *J. Cell. Biol.* 101, 540, 1985.
11. Gooding, L. R., Elmore, L. W., Tollefson, A. E., Brady, H. A., and Wold, W. S. M. A 14,700 MW protein from the E3 region of adenovirus inhibits cytolysis by tumor necrosis factor. *Cell* 53, 341, 1988.
12. Berkner, K. L. and Sharp, P. A. Generation of adenovirus by transfection of plasmids. *Nucleic Acids Res.* 11, 6003, 1983.
13. Kapoor, Q. S., Wold, W. S. M., and Chinnaduri, G. A non-essential glycoprotein is coded by early region E3 of adenovirus type 7. *Virology* 112, 780, 1981.
14. Lubeck, M. D., Davis, A. R., Chengalvala, M., Natuk, R. J., Morin, J. E., Molnar-Kimber, K., Mason, B. B., Bhat, B. M., Mizutani, S., Hung, P. P., and Purcell, R. H. Immunogenicity and efficacy testing in chimpanzees of an oral hepatitis B vaccine based on live recombinant adenovirus. *Proc. Natl. Acad. Sci. USA* 86, 6763, 1989.
15. Saito, I., Oya, Y., Yamamoto, K., Yuasa, T., and Shimojo, H. Construction of a non-defective adenovirus type 5 bearing a 2.8 kilobase hepatitis B virus DNA near the right end of its genome. *J. Virol.* 54, 711, 1985.
16. Yamada, M., Lewis, J. A., and Grodzicker, T. Overproduction of the protein product of a non-selected foreign gene carried by an adenovirus vector. *Proc. Natl. Acad. Sci. USA* 82, 3567, 1985.
17. Bernker, K. L. and Sharp, P. A. Effect of the tripartite leader on synthesis of a non-viral protein in an adenovirus 5 recombinant. *Nucleic Acids Res.* 13, 841, 1984.
18. Mansour, S. L., Grodzicker, T., and Tjian, R. Downstream sequences affect transcription initiation from the adenovirus major late promoter. *Mol. Cell. Biol.* 6, 2684, 1986.
19. Hanahan, D. and Gluzman, Y. Rescue of functional replication origins from embedded configuration in a plasmid carrying the adenovirus genome. *Mol. Cell. Biol.* 4, 302, 1984.

20. Wain-Hobson, S., Sonigo, P., Danos, O., Cole, S., and Alizon, M. Nucleotide sequence of the AIDS virus, LAV. *Cell* 40, 9, 1985.
21. Knight, D. M., Flomerfelt, F. A., and Ghrayeb, J. Expression of the *art*/*urs* protein of HIV and study of its role in viral envelope synthesis. *Science* 236, 837, 1987.
22. Hadzopoulou-Cladaras, M., Felber, B. K., Cladaras, C., Athanassopoulos, A., Tse, A., and Pavlakis, G. N. The *rev* (*urs/art*) protein of human immunodeficiency virus type 1 affects viral mRNA and protein expression via a *cis*-acting sequence in the *env* region. *J. Virol.* 63, 1265, 1989.
23. Hammariskjold, M.-L., Heimer, J., Hammariskjold, B., Sangwan, I., Albert, L., and Rekosh, D. Regulation of human immunodeficiency virus *env* expression by the *rev* gene product. *J. Virol.* 63, 1959, 1989.
24. Goh, W. C., Sodroski, J. G., Rosen, C. A., and Haseltine, W. A. Expression of the *art* gene protein of human T-lymphotropic virus type III (HTLV-III) in bacteria. *J. Virol.* 61, 633, 1987.
25. Chanda, P. K., Ghrayeb, J., and Wong-Staal, F. Nonrandom distribution of antibodies to the TRS protein of human immunodeficiency virus in infected people with different clinical status. *AIDS Res. Hum. Retroviruses* 4, 11, 1988.
26. Cullen, B. R., Hauber, J., Campbell, K., Sodroski, J. G., Haseltine, W. A., and Rosen, C. A. Subcellular localization of the human immunodeficiency virus trans-acting *art* gene product. *J. Virol.* 62, 2498, 1988.
27. Malim, M. H., Hauber, J., Le, S. Y., Mizel, J. V., and Cullen, B. R. The HIV-1 *rev* transactivator acts through a structural target sequence to activate nuclear export of unspliced viral mRNA. *Nature* 338, 354, 1989.
28. Grantham, R., Gautier, C., Gouy, M., Jacobzone, M., and Mercier, R. Codon usage is a genome strategy modulated for gene expressivity. *Nucleic Acids. Res.* 9, 43, 1981.
29. Lifson, J. D., Feinberg, M. B., Reyes, G. R., Rabin, L., Banapour, B., Chakrabarti, S., Moss, B., Wong-Staal, F., Steimer, K. S., and Engleman, E. G. Induction of CD4 dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. *Nature* 323, 725, 1986.
30. Lifson, J. D., Reyes, G. R., McGrath, M. S., Stein, B. S., and Engleman, E. G. AIDS retrovirus induced cytopathicity: giant cell formation and involvement of CD4 antigen. *Science* 232, 1123, 1986.
31. Sodroski, J., Goh, W. C., Rosen, C., Campbell, K., and Haseltine, W. A. Role of the HTLV-III/LAV envelope in syncytium formation and cytopathicity. *Nature (London)* 322, 470, 1986.
32. Lieber, M., Smith, B., Szakal, A., Nelson-Rees, W., and Todaro, G. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int. J. Cancer* 17, 62, 1976.
33. Top, F. H., Jr., Grossman, R. A., Bartelloni, P. J., Segal, H. E., Dudding, B. A., Russell, P. K., and Buescher, E. L. Immunization with live types 7 and 4 adenovirus vaccines. I. Safety, infectivity, antigenicity, and potency of adenovirus type 7 vaccine in humans. *J. Inf. Dis.* 124, 148, 1971.
34. Rice, N. R., Copeland, T. D., Simeck, S., Orszulán, S., and Gilden, R. V. Detection and characterization of the protein encoded by the *v-rel* oncogene. *Virology* 149, 217, 1986.
35. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* 227, 680, 1970.

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